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Award Number: W81XWH-12-1-0165

TITLE: Disparities in Intratumoral Steroidogenesis

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CONTRACTING ORGANIZATION: Children's Hospital Corporation
Boston MA 02115-5724

REPORT DATE: July 2013

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
July 2013	Annual Report	15 June 2012- 14 June 2013

4. TITLE AND SUBTITLE

Disparities in Intratumoral Steroidogenesis

5a. CONTRACT NUMBER
W81XWH-12-1-0165

5b. GRANT NUMBER
W81XWH-12-1-0165

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Children's Hospital Corporation, The
Boston MA 02115-5724

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Prostatic adenocarcinoma (PC) is the most common form of non-cutaneous cancer and second most lethal cancer in American men but demonstrates tremendous disparity in both incidence and severity between African American men (AAM) and Caucasian men (CM). We have identified *prostatic intratumoral steroidogenesis* as a biological factor that may explain some or much of the disparity in lethal PC rates between AAM and CM. We proposed testing this hypothesis by examining intratumoral steroidogenesis in the prostates of men following radical prostatectomy and *in vivo* model systems. In this project period we have finished our initial round of *in vivo* modeling and have demonstrated that hypercholesterolemia contributes to prostate tumor growth in our model mimicking the human patient situation in which androgen deprivation therapy (castration) is applied after tumor initiation. End point testing is currently underway and we anticipate finding excess androgen and nuclear AR in tumors undergoing relapse in hypercholesterolemic mice. Blinded analysis is on-going and will be unblinded as soon as all end point data has been collected. The final data are anticipated to reveal that, as hypothesized, hypercholesterolemia contributes to faster relapse after castration and increases intratumoral steroidogenesis.

15. SUBJECT TERMS
Hypercholesterolemia, castration, ADT, intratumoral steroidogenesis, androgen

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	35	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Introduction:

Prostatic adenocarcinoma (PC) is the most common form of non-cutaneous cancer and second most lethal cancer in American men but demonstrates tremendous disparity in both incidence and severity between African American men (AAM) and Caucasian men (CM). AAM have an incidence rate of 231.9 PCs/100,000 men whereas CM have an incidence rate of 146.3 PCs/100,000 men, a ratio of 1.58 AAM/CM, while the mortality rate for AAM is 56.3/100,000 and for CM is 23.6/100,000 men, a ratio of 2.39 AAM/CM[(1)]. These data show that AAM not only have a disproportionate incidence rate, but their mortality rate is almost 2.5X higher, suggesting that AAM are more likely than CM to have their cancer progress to advanced, fatal disease. The reasons for the high degree of disease burden in AAM are unknown, but may stem from biological, economic, psychological and sociological origins. Low socioeconomic status, absence of health insurance, poor access to health care, and lack of a regular primary care physician are substantial barriers to PC screening and early diagnosis[(2)]. These factors are likely responsible for some of the unequal disease burden between AAM vs. CM, but it is also apparent that there is a biological basis for the PC risk discrepancy. We have identified *prostatic intratumoral steroidogenesis* as a biological factor that explains some or much of the disparity in lethal PC rates between AAM and CM. In addition, circulating cholesterol has been identified in pre-clinical and epidemiological studies as a likely promoting factor in PC progression[(3-17)]. We hypothesize that serum cholesterol level affects tumor steroidogenesis by both serving as a rate-limiting metabolite precursor of intratumoral androgen synthesis, as well as a steroidogenic pathway agonist. We predict that we will find higher androgen levels in the tumors of men with higher levels of cholesterol. We further hypothesize that for any given serum cholesterol level, AAM will have higher tumor steroidogenesis. Using *in vivo* model systems we are testing whether therapeutically targeting cholesterol will reduce intratumoral steroidogenesis. This project specifically focuses on the effectiveness of cholesterol targeting as a means of delaying disease progression in the context of castration resistant prostate cancer, which is uniformly fatal.

Body:

Task 1. Determine whether there is a disparity in level of steroidogenic enzymes and androgens between the prostatic tumors of African American and Caucasian men (1-24 months). This task requires recruitment of patients, collecting patient serum, serology including cholesterol measures, radical prostatectomies or excision of metastatic lesions (Duke site), shipment of frozen tissue to the Solomon lab (Children's Hospital Boston, CHB, site), for analysis of androgens and steroidogenic enzymes.

To accomplish task 1 a new human subjects protocol (key parts are excerpted below) at Duke University Medical Center required institutional approval. A full copy of the current draft protocol is amended to this progress report.

1.0 STUDY DESIGN

This is a prospective study with no clinical intervention. Eligible patients will include those undergoing a radical prostatectomy regardless of disease risk or men undergoing excision of tissue for CRPC progression. Accrual will occur at both Duke University and the Durham VA. We anticipate enrolling 120 men undergoing a radical prostatectomy and 20 men undergoing excisional biopsy for CRPC progression over 2 years. Of the 120 men undergoing a radical prostatectomy, we anticipate only 30 will come from the Durham VA and of the 20 men undergoing excisional biopsy, we anticipate 5 coming from the Durham VA. After providing written consent, a blood sample, anthropomorphic measures, and basic medical history will be obtained prior to surgery. At the time of surgery, a sample of the excised tissue (either radical prostatectomy or excisional biopsy tissue) will be frozen and sent to Dr. Keith Solomon at Boston Children's Hospital for analyses to measure tissue androgen levels and expression of steroidogenic enzymes. All tissue samples will be sent to Dr. Solomon

and will be labeled only with unique subject number and date of surgery. Results from the tissue analyses will be sent to Duke for statistical analyses under the direction of Dr. Maragatha Kuchibhatla from Duke University.

2.0 ELIGIBILITY CRITERIA

2.1 Inclusion Criteria

1. Pathologically confirmed adenocarcinoma of the prostate
2. Elected primary radical prostatectomy or undergoing excision of tissue for CRPC progression including TURP
3. Race is either African-American or Caucasian
4. Evidence of a personally signed and dated informed consent document indicating that the subject has been informed of all pertinent aspects of the study.

2.2 Exclusion Criteria for Men in the Radical Prostatectomy Cohort

1. History of ever receiving hormone or antiandrogen therapy (e.g. finasteride, dutasteride, Avodart)
2. Prior prostate radiotherapy (external beam or brachytherapy) or cryotherapy

2.3 Exclusion Criteria for Men Undergoing Excision of Tissue for CRPC Progression

1. Unable to provide written informed consent

2.4 Withdrawal Criteria

Subjects who do not undergo radical prostatectomy or excision of tissue for any reason will be deemed non-evaluable and no further follow up will be collected. These subjects will be replaced.

3.0 STUDY PROCEDURES

5.1 Visit Schedule

Table 1: Evaluation and visit schedule

Examination	Screen	Study Visit	Tissue Collection
	D-60 to -1	D-60 to -1	Day of Surgery (D0)
Consent	X	(X)	
Eligibility	X	(X)	
Medical History and Demographics		X	
PSA		X	
Anthropometric Measures[1]		X	
Testosterone		X	
Free Testosterone		X	
Lipid Panel		X	
SHBG		X	

Prostatectomy or excision of CRPC progression[2]			X

[1] Height, weight, and waist circumference will be measured and collected
[2] With tissue procurement for molecular assessments

5.2 Screening and Study Visit

For enrollment at Duke University, prior to undergoing any study-specific procedures, patients must read and sign the IRB-approved informed consent form.

For enrollment at the VA, a HIPAA waiver will allow the study coordinator to perform the initial screening for eligibility among men undergoing a radical prostatectomy or excisional biopsy for CRPC progression. The study coordinator will use the computerized medical records system (CPRS) to ensure patients meet the inclusion/exclusion criteria stated in the protocol. Upon determining patient eligibility, the study coordinator will first speak with the patient at his pre-op appointment and attempt to consent him. If the subject is interested in participating, he can either complete the study procedures that day or the study coordinator can schedule a screening visit in the near future, as long as it is within the protocol specified window (Table 1). Documentation of the consent process and a copy of the signed consent will be maintained in the patient's medical record.

All study procedures are permitted within the window frame indicated in Table 1. The screening and study visit may be combined as the same visit.

The following procedures will be completed for this study:

1) Anthropometric measures: Height, weight, and waist circumference measurements will be completed.
2) Blood collection: Blood will be drawn and processed at the CLIA-certified Duke clinical laboratories for analysis of testosterone, free testosterone, PSA, lipid panel, and SHBG. Blood drawn at the VA will be processed at the VA.
3) Medical history and demographics: Obtain medical history and demographic information from patient and via electronic medical records.
4) Medical record follow-up: Outcome data (such as but not limited to PSA recurrence, time to recurrence, additional treatment, metastatic disease, mortality, and cause of death) will also be collected. This involves research staff following patients through electronic medical records or phone follow up until death.

5.3 Day of Surgery

The following will be performed on the day of surgery:

1) Either radical prostatectomy or biopsy for excision of tissue for CRPC progression, which the patient is scheduled for – this is not research.
2) Tissue collection at the time of surgery/biopsy for pathologic assessment and research assessment.

Tissue samples will be obtained immediately after removal of the prostate or excisional biopsy (fixation should occur within 30 minutes of resection)
.
For radical prostatectomy, a minimum of 6 core biopsies will be taken from tumor rich areas of the prostate identified from the biopsy pathology report, and from tumor-involved prostate if visible. An additional 2 cores of normal tissue will also be taken. The cores will be placed in tubes and snap frozen (no OCT).

For excisional biopsies of CRPC lesions, a portion of the tissue will be taken, placed in a tube and snap frozen (no OCT).

5.3.1 Tissue Procurement at Duke

Tissue collected from Duke subjects will be collected under the DUHS Biospecimen Repository and Processing Core (Pro00035974). Tissue will be processed and stored according to the BRPC protocol until shipped to Dr. Solomon.

5.3.2 Tissue Procurement at Durham VAMC

Tissue collected from Durham VAMC subjects will be done in conjunction with the Pathology Department at the Durham VAMC as not to interfere with appropriate pathological interpretation of the specimen for clinical purposes. Samples will be stored at Dr. Freedland's laboratory at Duke in the Medical Sciences Research Building until shipped to Dr. Solomon (stored <90 days). Unused samples will be returned to the Durham VA and stored either at the VA or at Duke in Dr. Freedland's laboratory under an off-site tissue bank waiver.

5.3.3 Shipping

Frozen prostate samples will be sent to Dr. Keith Solomon at Boston Children's Hospital. The tissue will be sent in batches by an overnight carrier to Dr. Keith Solomon at Boston Children's Hospital for analysis. Samples will be labeled with the study subject number and date of surgery. Frozen samples will be batch shipped (Monday and Tuesday shipment only) by overnight express for next day delivery on dry ice.

Frozen specimens will be shipped on dry ice to the following address:

Dr. Keith Solomon c/o Kristine Pelton
Boston Children's Hospital
Department of Urology
Enders 10
61 Binney st
Boston, MA 02115
Phone: 617-919-2937
Email: Kristine.pelton@childrens.harvard.edu

5.3.4 Tissue Analysis

Dr. Solomon will measure the level of steroidogenic/cholesterol sensitive enzymes using qPCR and western blotting: PSA, CYP17A1, CYP11A1, STaR, HSD3B1/2, HSD17B3, AKR1C1/2/3, 5RD5A1/2, HSD17B10, CYP19A1, ABCA1, ABCG1, ABCA7, CYP27A1 CYP7B1, LDLR & SR-B1, acyl-CoA cholesterol acyl transferase (ACAT), and HMG-CoA reductase. From the same tissue samples, Dr. Solomon will use mass spectrometry (MS) to measure tumor tissue levels of androstenedione, T, DHT, DHEA, and androstenediol. Finally, he will use immunoflouresence to analyze tumors for nuclear localization of the androgen receptor (AR).

Task 2. Using *in vivo* model systems we will determine if therapeutically targeting cholesterol alters intratumoral steroidogenesis. This task requires placing SCID mice on specialized diets that raise or reduce circulating cholesterol levels, implanting xenograft tumors, measuring tumor growth, and determining the effect of cholesterol raising and reducing on tumor growth and intratumoral steroidogenesis (CHB site) (months 1-36).

6 w old castrated and intact male SCID mice had their baseline cholesterol levels normalized by feeding them a low fat, no cholesterol (LFNC) diet for 2 w. The animals were then randomized into our unique diet scheme.

The basic design of our diet approach uses a defined low fat/no cholesterol [LFNC—equivalent of a normal chow diet which typically contains nominal (2 parts/million) cholesterol] and a high fat/high cholesterol diet (HFHC, w/o sodium cholate). The diets are balanced in micronutrients on a per-calorie basis (**see Table 2**), permitting us to use the diets isocalorically. Different gram amounts of each diet are fed to the mice, fixing the amount of calories/mouse. Our HFHC diet does not make mice obese, in fact they weigh the same as the mice

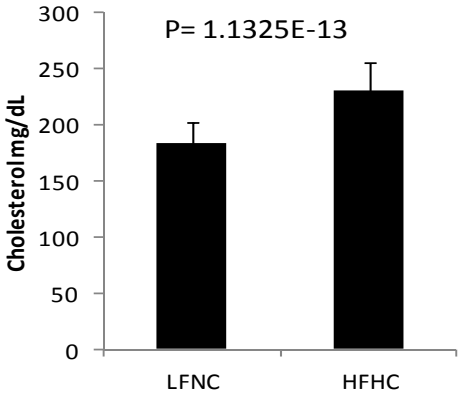

Figure 1. Cholesterol level in SCID mice. Mice were fed defined LFNC and HFHC diets (see Table 2) for 2 w and their cholesterol levels were measured. Data are presented as cholesterol level (mg/dL) ± SD vs. diet group. N=34-39 P=1.1325E-13 (Student's t-test).

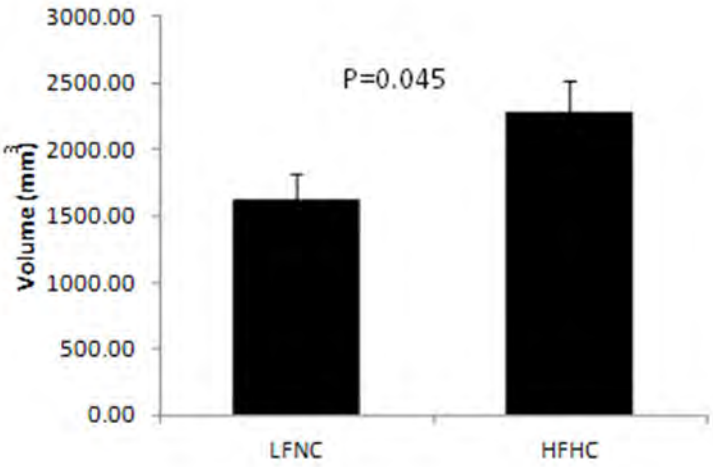

Figure 2. Tumor growth. SCID mice fed defined LFNC and HFHC diets were implanted with LNCaP tumors and tumor volume on the day of castration (24 d post implant) was measured. Data are presented as tumor volume (mm^3) ± SEM vs. diet group. N=25-28. P=0.045 (Student's t-test)

fed the LFNC diet, does not raise triglyceride (TG) levels in mice (not shown), but does raise cholesterol levels significantly[18-22] (**Fig 1**). The mice were then continued on their respective diets for 2 w, tested for cholesterol levels, and human PC cells were implanted subcutaneously as described[14,22]. Mice were bled (tail vein) every 3 d to monitor PSA, T (ELISA) and cholesterol levels (Infinity assay). 24 d after implantation most of tumors were palpable, with diet producing the expected effect on tumor growth (**Fig 2**). At that point, 2/3 of the mice were castrated (**Fig 3**). All mice were continued on their diet regimen. Castration caused tumor growth to attenuate, and within 2 d post castration further tumor growth could not be measured (not shown). 4 animals from each group were sacrificed on post-castration day 7, the remaining castrates were harvested 6 w post castration. This was an earlier than anticipated (**see Fig 4**) as attrition of the SCID mice due to lymphoma was higher than expected or ever noted by us in several our prior studies, some lasting several months[14,21-24]. Since our vendor has no explanation for this high non-protocol related death rate, we are switching our model to the Nude mouse (*Foxn1*$^{-/-}$) /xenograft model for future experiments. Nude mice, which are only athymic and lack, for the most part, the T cell arm of the immune system, as opposed to SCID mice that lack both B and T cells, do not experience high rates of spontaneous lymphoma, permitting us to complete the experiment as envisioned. At sacrifice the animals were be bled for cholesterol and other serological markers and tumors removed and used for endpoint testing. Tumors from this initial experiment are being analyzed blinded and we will not unblind the data until all endpoints have been measured. Our primary experimental endpoints are**: (1) Steroidogenic/ cholesterol sensitive enzymes.** We are using qPCR and western blotting to measure the level of steroidogenic proteins/enzymes: CYP17A1, CYP11A1, STaR, HSD3B1/2, HSD17B3, AKR1C1/2/3,

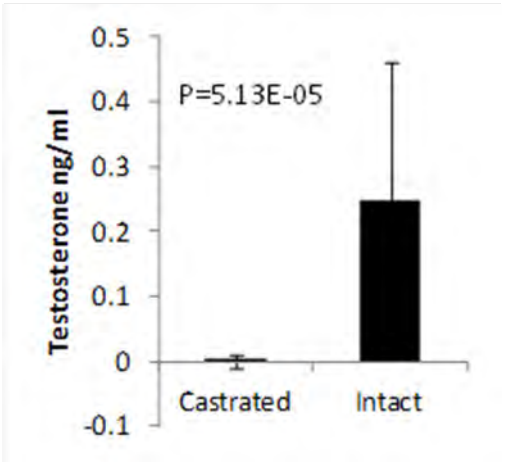

Figure 3. Testosterone level in castrated and intact mice. Mice fed defined diets and implanted with LNCaP tumors were either subjected to surgical castration or remained intact. At sacrifice terminal bleeds were performed and testosterone levels were measured by Elisa. Data are presented as testosterone (ng/ml) ± SD vs. group. N=8-13. P=5.13E-05 (Student's t-test).

5RD5A1/2, HSD17B10, and CYP19A1, similar to our published work[19]. We anticipate that elevated cholesterol level and castration will lead to an increase in the enzymes responsible for the synthesis of T and DHT in the tumor. This is a critical test of our hypothesis. We are also determining whether other proteins that are sensitive to changing cholesterol levels are regulated in this model: 1) cholesterol efflux transporters, ABCA1, ABCG1, and ABCA7; 2) Enzymes catalyzing the non-hepatic 'acid' pathway of bile acid synthesis CYP27A1 and CYP7B1[25]; 3) cholesterol receptors LDLR & SR-B1; 4) acyl-CoA cholesterol acyl transferase (ACAT) (cholesterol esterification); and 5) HMG-CoA reductase **(2) Androgen analysis.** We are also using mass spectrometry (MS) to determine whether castration and/or hyper- and hypocholesterolemia affect the tumor tissue levels of androstenedione, T, DHT, DHEA, and androstenediol using tumors generated in castrated and intact mice. **(3) AR analysis and activity level.** We are determining whether castration and/or hyper- and hypocholesterolemia affects the expression, and nuclear localization of the AR. Using IF we are analyzing tumors for nuclear localization of the AR and are determining by IF and Western the relative level of the AR in tumor xenografts. We anticipate that if the levels of androgens are increased by diet and/or castration, this will result in an increase in AR localization in nuclei. In contrast, hypocholesterolemia may decrease androgen synthesis and result in less nuclear AR. We are also interrogating the AR activity of the tumors by examining mRNA levels of PSA, a canonical AR-target gene.

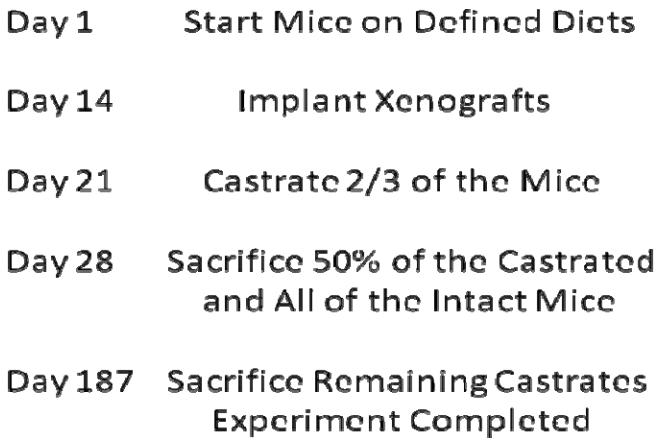

Figure 4. Flow chart of xenograft/castration experiments.

Diet Product #	Low-fat, no-chol (LFNC) (D12102C)		High-fat, with-chol (HFHC) (D12109C)	
%	gm	kcal	gm	kcal
Protein	19.2	20.0	22.5	20.0
Carbohydrate	67.3	70.0	45.0	40.0
Fat	4.3	10.0	20.0	39.9
Total		100.0		100.0
Kcal/gm	3.85		4.50	
Ingredient	gm	kcal	gm	kcal
Casein or Soy	200	800	200	800
L-cystine	3	12	3	12
Corn starch	375	1500	212	848
Maltodextrin 10	125	500	71	284
Sucrose	200	800	113	452
Cellulose, BW200	50	0	50	0
Soybean Oil	25	225	25	225
Coconut Oil, 101	0	0	0	0
Cocoa Butter	20	180	155	1395
Mineral Mix S10021	10	0	10	0
Dicalcium Phosphate	13	0	13	0
Calcium Carbonate	5.5	0	5.5	0
Potassium Citrate	16.5	0	16.5	0
Vitamin Mix V10001	10	40	10	40
Choline Bitartrate	2	0	2	0
Cholesterol	0	0	11.25	0
Red Dye, FD&C #40	0	0	0.05	0
Blue Dye, FD&C #1	0	0	0.05	0
Yellow Dye, FD&C #5	0.1	0	0	0
Total	1055.1	4057	901.85	4056

Table 2. Defined LFNC and HFHC diets.

Key Research Accomplishments:

- Demonstrated reproducibility concerning the effect of isocaloric low fat, no cholesterol and high fat, high cholesterol diets on circulating cholesterol levels in mice.
- Demonstrated reproducibility concerning the effect of isocaloric low fat, no cholesterol and high fat, high cholesterol diets on prostatic tumor growth, with high fat, high cholesterol diets causing accelerated tumor growth.
- Demonstrated the effect of isocaloric low fat, no cholesterol and high fat, high cholesterol diets on tumor growth in castrated mice.
- In the process of measuring the effect of diet on androgen level and AR activity in tumor grown in castrated mice fed isocaloric low fat, no cholesterol and high fat, high cholesterol diets.
- Produced an approvable IRB protocol to allow the collection of prostates from African American and Caucasian men requiring radical prostatectomies for prostate cancer.

Reportable Outcomes:

1) Created protocol to establish a prostate cancer tissue collection indexed with serum cholesterol values.
2) Generated a collection of human tumor xenografts from castrated and intact hosts to determine the effect of hyper and hypocholesterolemia on intratumoral steroidogenesis.
3) Gave a seminar at Hampton University, a historically black college, on cholesterol and prostate cancer risk in part due to research support by this award.
4) Dr. Solomon received an offer of employment from Hampton University, in part due to the research support by this award.
5) Kristine Pelton (Solomon Lab Manager) was given a promotion based in part on her efforts supported by this award.

Conclusion:

We have begun the analysis of tumor xenografts from castrated mice fed defined diets and anticipate that, as hypothesized, a high fat, high cholesterol 'Western diet' will contribute to intratumoral androgen synthesis. Preliminary findings suggest that we will have a decisive and reportable outcome to these initial experiments as all measured perameters thus far suggest a successful experiment: 1) Our low fat, no cholesterol (LFNC) vs. high fat, high cholesterol (HFHC) diets produced the expected effects on serum cholesterol (Fig 1); 2) the LFNC vs. HFHC diet produced the expected effects on tumor growth prior to castration (Fig 2); and 3) castration caused circulating testosterone levels to be undetectable (Fig 3) and tumor growth to cease. We have also created a human subjects protocol that is being approved at Duke University Medical Center and we should begin to collect human prostates for analysis in the next few weeks and begin to generate the data required to address the question of whether there are differences between Caucasan and African-American men in their levels of intratumoral androgen and whether high levels of serum cholesterol influence these levels.

Literature Cited:

1. Jemal, A., Siegel, R., Xu, J., and Ward, E. Cancer statistics, 2010.*CA Cancer J Clin* 60, 277-300
2. Talcott, J. A., Spain, P., Clark, J. A., Carpenter, W. R., Do, Y. K., Hamilton, R. J., Galanko, J. A., Jackman, A., and Godley, P. A. (2007). Hidden barriers between knowledge and behavior: the North Carolina prostate cancer screening and treatment experience.*Cancer* 109, 1599-1606
3. Freeman, M. R., and Solomon, K. R. (2004). Cholesterol and prostate cancer.*J Cell Biochem* 91, 54-69
4. Platz, E. A., Leitzmann, M. F., Visvanathan, K., Rimm, E. B., Stampfer, M. J., Willett, W. C., and Giovannucci, E. (2006). Statin drugs and risk of advanced prostate cancer.*J Natl Cancer Inst* 98, 1819-1825
5. Platz, E. A., Clinton, S. K., and Giovannucci, E. (2008). Association between plasma cholesterol and prostate cancer in the PSA era.*Int J Cancer* 123, 1693-1698
6. Blais, L., Desgagne, A., and LeLorier, J. (2000). 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors and the risk of cancer: a nested case-control study.*Arch Intern Med* 160, 2363-2368
7. Bravi, F., Scotti, L., Bosetti, C., Talamini, R., Negri, E., Montella, M., Franceschi, S., and La Vecchia, C. (2006). Self-reported history of hypercholesterolaemia and gallstones and the risk of prostate cancer.*Ann Oncol*
8. Freeman, M. R., Solomon, K. R., and Moyad, M. (2006). Statins and the risk of cancer.*Jama* 295, 2720-2721; author reply 2721-2722
9. Murtola, T. J., Tammela, T. L., Lahtela, J., and Auvinen, A. (2007). Cholesterol-Lowering Drugs and Prostate Cancer Risk: A Population-based Case-Control Study.*Cancer Epidemiol Biomarkers Prev* 16, 2226-2232
10. Pedersen, T. R., Wilhelmsen, L., Faergeman, O., Strandberg, T. E., Thorgeirsson, G., Troedsson, L., Kristianson, J., Berg, K., Cook, T. J., Haghfelt, T., Kjekshus, J., Miettinen, T., Olsson, A. G., Pyorala, K., and Wedel, H. (2000). Follow-up study of patients randomized in the Scandinavian simvastatin survival study (4S) of cholesterol lowering.*Am J Cardiol* 86, 257-262
11. Platz, E., Leitzmann, M., Visvanathan, K., and al., e. (2005). Cholesterol-lowering drugs including statins and the risk of prostate cancer in a large prospective cohort study. In Proc Amer Assoc Cancer Res.
12. Platz, E. A., Clinton, S. K., and Giovannucci, E. (2006). Association between plasma cholesterol and prostate cancer. In AACR Frontiers in Cancer Prevention Research (Boston, MA).
13. Platz, E. A., Leitzmann, M. F., Visvanathan, K., Stampfer, M. J., Willett, W. C., Rimm, E. B., and Giovannucci, E. (2005). Cholesterol-lowering drugs including statins and the risk of prostate cancer in a large prospective cohort study.*AACR Meeting Abstracts* 2005, 1034-a-
14. Zhuang, L., Kim, J., Adam, R. M., Solomon, K. R., and Freeman, M. R. (2005). Cholesterol targeting alters lipid raft composition and cell survival in prostate cancer cells and xenografts.*J Clin Invest* 115, 959-968
15. Zhuang, L., Lin, J., Lu, M. L., Solomon, K. R., and Freeman, M. R. (2002). Cholesterol-rich lipid rafts mediate akt-regulated survival in prostate cancer cells.*Cancer Res* 62, 2227-2231
16. Solomon, K. R., and Freeman, M. R. (2008). Do the cholesterol-lowering properties of statins affect cancer risk?*Trends Endocrinol Metab* 19, 113-121
17. Iso, H., Ikeda, A., Inoue, M., Sato, S., and Tsugane, S. (2009). Serum cholesterol levels in relation to the incidence of cancer: the JPHC study cohorts.*Int J Cancer* 125, 2679-2686
18. Kim, J., Di Vizio, D., Kim, T. K., Kim, M., Pelton, K., Clinton, S. K., Hai, T., Hwang, D., Solomon, K. R., and Freeman, M. R. (2012). The response of the prostate to circulating cholesterol: activating transcription factor 3 (ATF3) as a prominent node in a cholesterol-sensing network.*PloS One* 7, e39448
19. Mostaghel, E. A., Solomon, K. R., Pelton, K., Freeman, M. R., and Montgomery, R. B. (2012). Impact of circulating cholesterol levels on growth and intratumoral androgen concentration of prostate tumors.*PloS One* 7, e30062
20. Pelton, K., Di Vizio, D., Insabato, L., Schaffner, C. P., Freeman, M. R., and Solomon, K. R. (2010). Ezetimibe reduces enlarged prostate in an animal model of benign prostatic hyperplasia.*J Urol* 184, 1555-1559

21. Pelton, K., Krieder, J., Joiner, D., Freeman, M. R., Goldstein, S. A., and Solomon, K. R. (2012). Hypercholesterolemia promotes an osteoporotic phenotype.*Am J Pathol* 181, 928-936
22. Solomon, K. R., Pelton, K., Boucher, K., Joo, J., Tully, C., Zurakowski, D., Schaffner, C. P., Kim, J., and Freeman, M. R. (2009). Ezetimibe is an inhibitor of tumor angiogenesis.*Am J Pathol* 174, 1017-1026
23. Adam, R. M., Mukhopadhyay, N. K., Kim, J., Di Vizio, D., Cinar, B., Boucher, K., Solomon, K. R., and Freeman, M. R. (2007). Cholesterol sensitivity of endogenous and myristoylated Akt.*Cancer Res* 67, 6238-6246
24. Solomon, K. R., and Freeman, M. R. (2011). The complex interplay between cholesterol and prostate malignancy.*Urol Clin North Am* 38, 243-259
25. An, S., Jang, Y. S., Park, J. S., Kwon, B. M., Paik, Y. K., and Jeong, T. S. (2008). Inhibition of acyl-coenzyme A:cholesterol acyltransferase stimulates cholesterol efflux from macrophages and stimulates farnesoid X receptor in hepatocytes.*Exp Mol Med* 40, 407-417

DISPARITIES IN INTRATUMORAL STEROIDOGENESIS

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Table of Contents

1.0 BACKGROUND AND RATIONALE

1.1 Background

Prostatic adenocarcinoma (PCa) is the most common form of non-cutaneous cancer and second most lethal cancer in American men, with an incidence of 217,730 new cases, and more than 32,050 deaths in 2010 alone[1]. PCa is also a cancer which demonstrates tremendous disparity in both incidence and severity between African American men (AAM) and Caucasian men (CM). AAM have an incidence rate of 231.9 PCas/100,000 men whereas CM have an incidence rate of 146.3 PCas/100,000 men, a ratio of 1.58 AAM/CM, while the mortality rate for AAM is 56.3/100,000 and for CM is 23.6/100,000 men, a ratio of 2.39 AAM/CM[1]. These data show that AAM not only have a disproportionate incidence rate, but their mortality rate is almost 2.5X higher, suggesting that AAM are more likely than CM to have their cancer progress to advanced, fatal disease. The reasons for the high degree of disease burden in AAM are unknown, but may stem from biological, economic, psychological and sociological origins. Low socioeconomic status, absence of health insurance, poor access to health care, and lack of a regular primary care physician are substantial barriers to PCa screening and early diagnosis[21]. These factors are likely responsible for some of the unequal disease burden between AAM vs. CM, but it is also apparent that there is a biological basis for the PCa risk discrepancy. For example, we have shown in even in an equal-access medical center and when treated equally, AAM are more likely to have their cancers progress after adjusting for disease characteristics[22]. Moreover, multiple studies determined allelic variants in chromosomal regions, especially 3 separate regions (risk-regions) of chromosome 8q24 spanning ≈600kb are associated with a higher risk of PCa or of metastatic prostate disease in both CM and AAM[23-28]. Of particular interest is risk-region 2 in which the risk alleles are carried at a much higher frequency in AAM than in CM. None of the risk-regions are gene encoding and none reside within or near genes, suggesting that these genetic elements might modify PCa risk by altering vital pathways. Interestingly, 3 adjacent SNPs centromeric to risk-region 2 have been shown to be associated with circulating testosterone (T) levels[29], suggesting that this region may regulate steroidogenesis. Given our data and those of others that implicate intratumoral steroidogenesis as a critical aspect of PCa progression, the genetic linkage data suggesting that regulation of testosterone level as a potential point of elevated risk in AAM, and the higher androgen receptor (AR) expression levels in AAM[30], Dr. Solomon has identified *prostatic intratumoral steroidogenesis* as a biological factor that may explain some or much of the disparity in lethal PCa rates between AAM and CM. These observations stem directly from our work and that of others that suggests that prostate tumor cells in human patients synthesize androgens *de novo* directly from cholesterol[2-19]. A prior study of intratumoral androgen levels demonstrated no difference between AAM and CM[31]. This prior study used insensitive ELISA assays, and biased the results toward the null by assigning the assay's lower limit of detection to any sample that returned a 'zero' reading, a technique that makes this prior analysis inconclusive. Given the importance of the question, a fresh look at potential racial disparity in intratumoral steroidogenesis using modern sensitive techniques is needed. In the current study we will use tandem mass spectrometry that is sensitive in the 100 femtomol/gm tissue range.

***Intratumoral Androgen Synthesis*:** Prostatic tumors respond to circulating androgens through the action of the AR, which drives the proliferation of PCa cells, even under conditions of hormone suppression during late-stage disease[13,18,32]. Androgen Deprivation Therapy (ADT) is

the primary treatment strategy for advanced metastatic PCa[13,33]. Despite widespread early responses, PCa almost invariably becomes "castration resistant" (castration resistant prostate cancer; CRPC) and the tumor cells continue to grow despite systemic castrate levels of androgens[13,18]. In this phase of disease, tumors become more aggressive. There are several theories about how this castration-resistant phenotype comes about: 1) gene amplification and/or mutation of the AR, allowing the receptor to be sensitive to low levels of androgen[34-40]; 2) residual androgen production from the adrenals[14]; and 3) promiscuous receptor-ligand interactions[36,41]. Although it was 30 years ago that it was first reported[42] that sufficient androgen to drive the AR remained in the prostate after ADT[42,43], the significance of this observation was partially obscured, as other work appeared to demonstrate an absence of functional androgen in men with CRPC[44]. The combination of castration with an anti-androgen is currently commonly used to treat advanced PCa. Further evidence that CRPC is still driven, at least in part, by androgen is provided by results of a phase III trial using the CYP17 inhibitor, abiraterone, in men with CRPC showing a significant overall survival benefit[45]. Several groups have presented data which suggest that PCa cells in men receiving ADT have androgen levels high enough to activate the AR[3-5,7,9,11,12,16,17,19,42,44,46-50]. <u>The mechanism responsible for maintenance of functional tissue levels of T and DHT in CRPC is unknown.</u>

Recently, several lines of evidence are converging on the hypothesis that PCa cells synthesize their own androgens, including in the castrate environment[2,15,50]. Our data (Figs 1&2, see below) are consistent with this idea. Locke et al.[15] demonstrated that ***<u>all</u>*** of the enzymes necessary for *de novo* androgen synthesis are expressed in LNCaP tumor xenografts, and that androgen-starved PCa cells are capable of synthesizing DHT from acetic acid, suggesting that the entire pathway from acetate→cholesterol→DHT is intact in this model system[15]. Montgomery et al.[50] demonstrated that the full complement of enzymes comprising the steroidogenic pathways are present in the majority of ***<u>human</u>*** primary and metastatic PCas examined[50], implying that *de novo* androgen synthesis is not merely an experimental phenomenon, but rather a potential underlying cause of disease progression in the hormone-repressed state. In addition, a very recent report demonstrates that PCa bone metastases have higher concentrations of cholesterol over the levels found in normal bone and in bone metastases from other malignancies[51]. Increased cholesterol along with the ability to synthesize androgen fundamentally support our hypothesis that *de novo* steroidogenesis occurs in CRPC.

The ability of PCa cells to synthesize androgen from cholesterol may actually increase following hormone suppression because castration increases serum cholesterol levels[49,52-54], as well as cholesterol synthesis in experimental prostatic tumors[8]. Dr. Solomon has noted this increase in serum cholesterol in castrated mice (not shown). Our studies of castrated mice show that hypercholesterolemia accelerates the growth of PCa xenografts in the hormone-suppressed condition (Fig 3), and suggests that hypercholesterolemia contributes to androgen synthesis in prostate tumors. Interestingly, in contrast to tumors grown in hormonally intact mice (Figs 1&2), which have T >DHT, tumors grown in castrates have DHT >T.

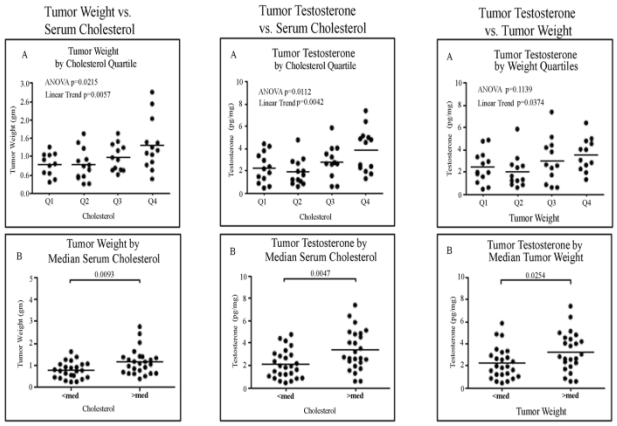

Fig. 1. The relationship between circulating cholesterol level, tumor growth and tumor testosterone (T). Tumors grown in mice that were either hypo-, normo-, or hypercholesterolemic were weighed and portions were analyzed by MS for the presence of T. Circulating cholesterol in the mice was determined by Infinity assay. In row A the cohort is divided by quartile of circulating cholesterol level (2 left most panels) and by quartile of tumor weight (right most panel). In row B the cohort is divided into < or > median value (in the left most 2 panels it is circulating cholesterol, in the right most panel it is tumor weight). Data are graphed as dot plots (each dot representing a different mouse or tumor). ANOVA indicated significant effects of serum cholesterol on tumor weight, and on T levels. ANOVA also indicated a significant effect of T on tumor growth when analyzed by < > median. N=11-13 for each quartile in row A and N=24-26 < > median in row B.

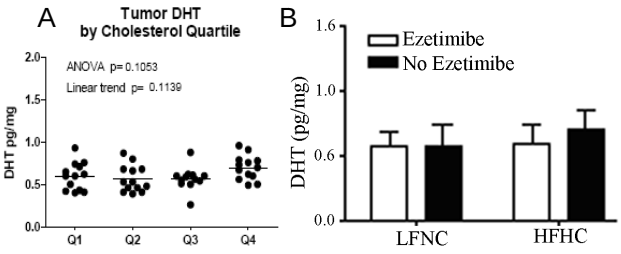

Fig 2. Tumor DHT levels are increased in the tumors of hypercholesterolemic mice. Mice were fed either a HFHC or LFNC diet ±ezetimibe and implanted with tumors which were harvested 14d after first appearance. Tumors were removed and analyzed for DHT levels by MS. Data are plotted in A as DHT (pg/mg) level vs. quartile of cholesterol. In B, data are plotted as DHT vs. diet group ±SD. Data were analyzed by ANOVA, which indicated that in A the data had not yet reached, but was nearing significance (p=0.10, p for trend 0.11). Analysis of diet/DHT data indicated that the levels in tumors from hypercholesterolemic diets trended higher (p=0.054). N=11-13.

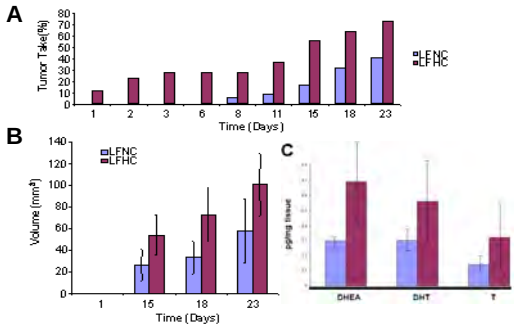

Fig 3. Tumor growth in castrated mice. Castrated mice were fed normo- (LFNC) or hypercholesterolemic diets (LFHC) for 80d, LNCaP cells were implanted and the mice continued on the diets for 22d. **(A) Tumor take**. The # of tumors in each diet group were counted & data plotted as tumor take (% of implantation sites) vs. Time (d). Significance was determined by logistic regression analysis. At all points where tumors were present, the 2 cohorts differed significantly. P<0.05;n=40/group. **(B) Longitudinal volume measurements.** Tumors were measured at various times starting at 1st appearance (d 1) & continued for 22d. Data are plotted as tumor volume (mm^3)/site vs. Time (days)±SE;n=40/group. (**C) Androgens in tumors.** Tumors from mice fed a LFNC or a LFHC diet were removed and portions of the tumor analyzed by MS. Data are presented as mean DHEA, DHT, or T levels (pg/mg tissue)±SE. n=6/group. These data are not significant.

Identifying Cholesterol as a Modifiable Risk Factor in Intratumoral Steroidogenesis: My laboratory has been investigating the causal connection between altered cholesterol homeostasis and PCa. Cholesterol accumulates in normal aging prostate tissue and in PCa[55-58]. **Importantly, epidemiologic and pre-clinical studies have found a correlation between serum cholesterol level and PCa incidence and/or progression**[55,58-71]. 'Western' diets, which are high in fat and cholesterol, have been linked to PCa, although the specific dietary components responsible for this association are incompletely understood[72-79]. Furthermore, several studies of cholesterol-lowering drugs (primarily HMG-CoA reductase inhibitors, a.k.a. statins) have found **an inverse association between statin use and PCa incidence and/or progression, including a significant reduction in advanced disease risk in men who have taken statins for ≥5 years**[59,61,63-66,68,80-89].

Dr. Solomon has shown hypercholesterolemia stimulates growth of LNCaP human PCa xenografts[69,90]. Tumors in the hypercholesterolemic environment accumulated more cholesterol in their membranes, exhibited less apoptosis, had enhanced Akt activation (a kinase linked to aggressive PCa)[91], and had more angiogenesis[69,90]. Dr. Solomon also showed hypocholesterolemia has the opposite effect, inhibiting prostatic tumor growth. In explaining these results, Dr. Solomon hypothesized that cholesterol might directly contribute to tumor growth by altering signal transduction pathways[55,69,70]. How can cholesterol affect tumor growth

so profoundly? Cholesterol makes up almost 1/3 of plasma membrane lipids. The effect of cholesterol on membrane organization is a feature of its unique chemistry, where the combination of the stiff, fused ring system and small head group affects cholesterol packing with other lipids and proteins. In mammalian cells, cholesterol content is a key determinant of membrane property and its concentration in cell membranes is tightly regulated, even as the external availability of cholesterol varies widely. While many studies including our own have demonstrated that the ability of cholesterol to organize membrane structure into liquid ordered domains affects signal transduction, including signaling by the epidermal growth factor receptor (EGFR) and Akt1[70,92], other explanations for the effect of hypercholesterolemia on PCa risk warrant careful consideration. In particular, one important new hypothesis is that **cholesterol affects PCa growth by serving as a precursor for intratumoral androgen synthesis.**

Cholesterol, Tumor Growth & Androgen Synthesis in the Mouse: Studying the *specific* role of cholesterol in murine models has been difficult to accomplish; serum cholesterol levels in normal mice cannot be reduced by statin drugs[58,93,94] and, as Dr. Solomon has written about extensively, studies using statins cannot be interpreted as showing a cholesterol effect because statins do not target cholesterol specifically[58,90]. Also, standard diets used for inducing hypercholesterolemia in mice contains sodium cholate, which is both hepatotoxic and unnecessary for raising cholesterol levels[90,95,96]. Studies of dietary fat and cholesterol often control for caloric effects of by balancing the control diets with added carbohydrates[95], making the mice hyperglycemic and raising insulin levels, or in other cases don't control for energy at all[97]. Given that carbohydrate restriction and the resultant lower serum insulin levels can also slow tumor growth[98,99], most diet studies are difficult to interpret.

Using novel diets and feeding strategies, Dr. Solomon created an innovative isocaloric diet approach in which Dr. Solomon determined the specific effects of cholesterol in mice by following 4 murine cohorts each with a different serum cholesterol level (≈140, 160, 180 & 200 mg/dL). As Dr. Solomon has shown[90], this approach permits us to study hyper and hypocholesterolemia simultaneously without affecting liver function, insulin levels, animal weight, or circulating steroid hormone levels. The basic design of our cholesterol-targeted approach combines a diet regimen with a pharmaceutical agent, ezetimibe (Zetia) that specifically targets cholesterol. In this scheme Dr. Solomon used a low fat/no cholesterol diet (LFNC) and a high fat/high cholesterol diet (HFHC) (w/o sodium cholate) ± ezetimibe (30mg/kg/d). Ezetimibe is an FDA approved drug that blocks cholesterol uptake in the gut, thereby lowering serum cholesterol levels. Ezetimibe is a specific antagonist of NPC1L1, the *bona fide* gut cholesterol transporter[100-103]. Ezetimibe has no known target other than NPC1L1, and NPC1L1 is expressed only in the gut, and in hepatocytes (in humans), but not by tumor cells. Treating PCa cells *in vitro* with ezetimibe has no affect on cell growth or survival (data not shown).

Having achieved the ability to specifically alter cholesterol levels isocalorically, Dr. Solomon created hypo, normo, and hypercholesterolemic groups and implanted mice with LNCaP cells[90]. As Dr. Solomon previously reported[90] mice fed the HFHC diet developed larger tumors, whereas mice fed the LFNC diet had smaller tumors. The addition of ezetimibe to either diet reduced tumor size. The combination of the LFNC diet + ezetimibe had the most significant effect on tumor growth (vs. the HFHC diet w/o ezetimibe). Statistical evaluation demonstrated that both

the diet (p=0.048) and ezetimibe (p=0.035) produced significant independent, additive, but not synergistic, effects on tumor growth (both volume & weight). Serology showed no liver dysfunction, no statistical differences in triglyceride (TG) levels (trending higher in the HFHC cohort), no detectable effect on serum T & no statistical differences in insulin or IGF-1 levels (not shown). ***Conclusion- circulating cholesterol directly contributes toward PCa growth independent of serum T.***

Dr. Solomon quantified the level of T (Fig 1) and DHT (Fig 2) in the tumors. To understand the complex relationship between cholesterol, tumor growth and T, Dr. Solomon analyzed the tumor data in aggregate, divided the samples in half or into quartiles of serum cholesterol or tumor weight and determined whether there were significant differences in the T levels between the groups. As demonstrated in Fig 1 these data suggest higher serum cholesterol level lead to higher intratumoral T and this may, in part, explain the relationship between higher cholesterol and tumor growth. As demonstrated in Fig 2, the difference in DHT levels trended higher in the highest cholesterol quartile, neared significance between HFHC and LFNC groups, and trended toward higher levels in the HFHC cohort. DHEA level trended similarly to T and androstenedione levels were not significantly different (not shown). ***Conclusion—Cholesterol may directly contribute to intratumoral androgen synthesis.***

1.2 Rationale

Prostatic adenocarcinoma (PCa) is the most common form of non-cutaneous cancer and second most lethal cancer in American men, with an incidence of 217,730 new cases, and more than 32,050 deaths in 2010 alone[1]. PCa is also a cancer which demonstrates tremendous disparity in both incidence and severity between Caucasian and African American men. African Americans have an incidence rate of 231.9 PCas/100,000 men whereas, Caucasians have an incidence rate of 146.3 PCas/100,000 men, a ratio of 1.58 AA/C, while the mortality rate for African Americans it's 56.3/100,000 and Caucasians is 23.6/100,000 men, a ratio of 2.39 AA/C. These data suggest that African American not only have a disproportionate incidence rate, but their mortality rate is almost 2.5 times higher, suggesting that African Americans are more likely than Caucasians to have their cancer progress to advanced, fatal disease. The reasons for the high degree of disease burden in African Americans is unknown, but may stem from biological, economic, psychological and sociological origins. Dr. Solomon has identified *prostatic intratumoral steroidogenesis* as a critical biological factor that may explain some or much of the disparity in lethal PCa rates between African American and Caucasian men. These observations stem directly from our own work and that of others that suggests that prostate tumor cells in human patients synthesize androgens *de novo* directly from cholesterol[2-19].

Based upon these findings, we **hypothesize** that dietary and circulating cholesterol contribute disproportionately to intratumoral androgen synthesis and to the development of castration-resistant PCa (CRPC) in African American men. We aim to test this in a prospective observational study by collecting serum blood measured for cholesterol and other factors and correlate this with tumor expression of steriodogenic enzymes and tumor androgen levels.

2.0 OBJECTIVES

2.1 Primary Objective

Determine whether there is a correlation between serum cholesterol and levels of tumor androgens and steroidogenic enzymes and whether these correlations differ in African American men.

2.2 Secondary Objective

Determine whether there is a correlation between total testosterone, free testosterone, or sex hormone binding globulin (SHBG) and levels of tumor androgens and steroidogenic enzymes and whether these correlations differ in African American men.

3.0 STUDY DESIGN

This is a prospective study with no clinical intervention. Eligible patients will include those undergoing a radical prostatectomy regardless of disease risk or men undergoing excision of tissue for CRPC progression. Accrual will occur at both Duke University and the Durham VA. We anticipate enrolling 120 men undergoing a radical prostatectomy and 20 men undergoing excisional biopsy for CRPC progression over 2 years. Of the 120 men undergoing a radical prostatectomy, we anticipate only 30 will come from the Durham VA and of the 20 men undergoing excisional biopsy, we anticipate 5 coming from the Durham VA. After providing written consent, a blood sample, anthropomorphic measures, and basic medical history will be obtained prior to surgery. At the time of surgery, a sample of the excised tissue (either radical prostatectomy or excisional biopsy tissue) will be frozen and sent to Dr. Keith Solomon at Boston Children's Hospital for analyses to measure tissue androgen levels and expression of steroidogenic enzymes. All tissue samples will be sent to Dr. Solomon and will be labeled only with unique subject number and date of surgery. Results from the tissue analyses will be sent to Duke for statistical analyses under the direction of Dr. Maragatha Kuchibhatla from Duke University.

4.0 ELIGIBILITY CRITERIA

4.1 Inclusion Criteria

1. Pathologically confirmed adenocarcinoma of the prostate
2. Elected primary radical prostatectomy or undergoing excision of tissue for CRPC progression including TURP
3. Race is either African-American or Caucasian
4. Evidence of a personally signed and dated informed consent document indicating that the subject has been informed of all pertinent aspects of the study.

4.2 Exclusion Criteria for Men in the Radical Prostatectomy Cohort

1. History of ever receiving hormone or antiandrogen therapy (e.g. finasteride, dutasteride, Avodart)
2. Prior prostate radiotherapy (external beam or brachytherapy) or cryotherapy

4.3 Exclusion Criteria for Men Undergoing Excision of Tissue for CRPC Progression

1. Unable to provide written informed consent

4.4 Withdrawal Criteria

Subjects who do not undergo radical prostatectomy or excision of tissue for any reason will be deemed non-evaluable and no further follow up will be collected. These subjects will be replaced.

5.0 STUDY PROCEDURES

5.1 Visit Schedule

Table 1: Evaluation and visit schedule

Examination	Screen	Study Visit	Tissue Collection
	D-60 to -1	D-60 to -1	Day of Surgery (D0)
Consent	X	(X)	
Eligibility	X	(X)	
Medical History and Demographics		X	
PSA		X	
Anthropometric Measures[1]		X	
Testosterone		X	
Free Testosterone		X	
Lipid Panel		X	
SHBG		X	
Prostatectomy or excision of CRPC progression[2]			X

[1] Height, weight, and waist circumference will be measured and collected
[2] With tissue procurement for molecular assessments

5.2 Screening and Study Visit

For enrollment at Duke University, prior to undergoing any study-specific procedures, patients must read and sign the IRB-approved informed consent form.

For enrollment at the VA, a HIPAA waiver will allow the study coordinator to perform the initial screening for eligibility among men undergoing a radical prostatectomy or excisional biopsy for CRPC progression. The study coordinator will use the computerized medical records system (CPRS) to ensure patients meet the inclusion/exclusion criteria stated in the protocol. Upon determining patient eligibility, the study coordinator will first speak with the patient at his pre-op appointment and attempt to consent him. If the subject is interested in participating, he can either complete the study procedures that day or the study coordinator can schedule a screening visit in the near future, as long as it is within the protocol specified window (Table 1). Documentation of the consent process and a copy of the signed consent will be maintained in the patient's medical record.

All study procedures are permitted within the window frame indicated in Table 1. The screening and study visit may be combined as the same visit.

The following procedures will be completed for this study:

1) Anthropometric measures : Height, weight, and waist circumference measurements will be completed.
2) Blood collection: Blood will be drawn and processed at the CLIA-certified Duke clinical laboratories for analysis of testosterone, free testosterone, PSA, lipid panel, and SHBG. Blood drawn at the VA will be processed at the VA.
3) Medical history and demographics: Obtain medical history and demographic information from patient and via electronic medical records.
4) Medical record follow-up: Outcome data (such as but not limited to PSA recurrence, time to recurrence, additional treatment, metastatic disease, mortality, and cause of death) will also be collected. This involves research staff following patients through electronic medical records or phone follow up until death.

5.3 Day of Surgery

The following will be performed on the day of surgery:

1) Either radical prostatectomy or biopsy for excision of tissue for CRPC progression, which the patient is scheduled for – this is not research.
2) Tissue collection at the time of surgery/biopsy for pathologic assessment and research assessment.

Tissue samples will be obtained immediately after removal of the prostate or excisional biopsy (fixation should occur within 30 minutes of resection)
.
For radical prostatectomy, a minimum of 6 core biopsies will be taken from tumor rich areas of the prostate identified from the biopsy pathology report, and from tumor-involved prostate if visible. An additional 2 cores of normal tissue will also be taken. The cores will be placed in tubes and snap frozen (no OCT).

For excisional biopsies of CRPC lesions, a portion of the tissue will be taken, placed in a tube and snap frozen (no OCT).

5.3.1 Tissue Procurement at Duke

Tissue collected from Duke subjects will be collected under the DUHS Biospecimen Repository and Processing Core (Pro00035974). Tissue will be processed and stored according to the BRPC protocol until shipped to Dr. Solomon.

5.3.2 Tissue Procurement at Durham VAMC

Tissue collected from Durham VAMC subjects will be done in conjunction with the Pathology Department at the Durham VAMC as not to interfere with appropriate pathological interpretation of the specimen for clinical purposes. Samples will be stored at Dr. Freedland's laboratory at Duke in the Medical Sciences Research Building until shipped to Dr. Solomon (stored <90 days). Unused samples will be returned to the Durham VA and stored either at the VA or at Duke in Dr. Freedland's laboratory under an off-site tissue bank waiver.

5.3.3 Shipping

Frozen prostate samples will be sent to Dr. Keith Solomon at Boston Children's Hospital. The tissue will be sent in batches by an overnight carrier to Dr. Keith Solomon at Boston Children's Hospital for analysis. Samples will be labeled with the study subject number and date of surgery. Frozen samples will be batch shipped (Monday and Tuesday shipment only) by overnight express for next day delivery on dry ice.

Frozen specimens will be shipped on dry ice to the following address:

Dr. Keith Solomon c/o Kristine Pelton
Children's Hospital Boston
Department of Urology
Enders 10
61 Binney st
Boston, MA 02115
Phone: 617-919-2937
Email: Kristine.pelton@childrens.harvard.edu

5.3.4 Tissue Analysis

Dr. Solomon will measure the level of steroidogenic/cholesterol sensitive enzymes using qPCR and western blotting: PSA, CYP17A1, CYP11A1, STaR, HSD3B1/2, HSD17B3, AKR1C1/2/3, 5RD5A1/2, HSD17B10, CYP19A1, ABCA1, ABCG1, ABCA7, CYP27A1 CYP7B1, LDLR & SR-B1, acyl-CoA cholesterol acyl transferase (ACAT), and HMG-CoA reductase. From the same tissue samples, Dr. Solomon will use mass spectrometry (MS) to measure tumor tissue levels of androstenedione, T, DHT, DHEA, and androstenediol. Finally, he will use immunoflouresence to analyze tumors for nuclear localization of the androgen receptor (AR).

6.0 STATISTICAL METHODS

Descriptive summary statistics (e.g., mean ±sd, etc.) will be provided for each quantitative endpoint related to serum cholesterol and tissue androgen levels as a whole and stratified by race. Box-Cox transformations will be used for each endpoint to evaluate whether Gaussian assumptions are met. The association between measurements will be evaluated using Pearson's correlation coefficient; the non-parametric Spearman rank correlation will be used when normality assumptions are not met. Correlation coefficients will be reported with 95% CIs. N=120 paired measurements will provide 93% power to reject an unacceptable correlation of $r<0.4$ when the true correlation is $r=0.612$, indicating a moderate or stronger level of correlation20. Further, regression models will be used in an exploratory manner to characterize the relationship among variables including linear & polynomial least-squares models, knotted and smooth splines, to evaluate the dynamic range and any threshold values in any non-linear associations. Cross-validation and resampling methods will be used to evaluate the relative performance of models to avoid over-fitting. To evaluate whether the correlation between cholesterol level and intratumoral steroidogenesis is the same for AAM and CM, we will use the general linear model to test whether there is a multiplicative interaction setting a two-sided alpha = 0.05. Under a Gaussian simulation of the alternative hypothesis that $r=0.612$ overall, there would be 85% power to detect a difference of $r = 0.40$ in AA and $r = 0.824$.

For the CRPC lesions, the limited sample size is determined for detecting an overall association and prevents a definitive study of the interaction with race, however, descriptive statistics will be used to characterize the agreement seen in each racial cohort, and if a non-significant, but clinical interesting difference in correlation is observed, predictive power calculations will be used to design future studies only. It is possible cholesterol levels at enrollment, especially for the CRPC lesions, may not reflect the environment in which the tumor developed. To account for this, we will also explore the link between pre-diagnostic cholesterol levels and tissue levels of androgens and steroidogenic enzymes.

7.0 DISCOMFORTS AND RISKS

Risks and side effects related to the procedures associated with this study include:

- Radical Prostatectomy or excisional biopsy: The procedure is not experimental. The procedure they are undergoing is a part of routine care and is not part of this study. Consent for this procedure and risks will be covered under a separate surgical consent form.

Medical History/Demographic Information/Accessing Medical Records: There are no physical risks involved with collecting or accessing the Durham VA's electronic medical records system, however there are risks associated with loss of confidentiality of sensitive information. Patients' information that is collected from CPRS will be housed on the secure Durham VA server that is only accessible to IRB-approved study personnel. Patients will be assigned a unique study ID which will be used when samples or data are given to Dr. Keith

Solomon or Dr. Maragatha Kuchibhatla. Tissue samples going to Dr. Keith Solomon will also include the date of surgery. The key to the code will reside on the Durham VA server.

- Blood tests: Risks associated with drawing blood from a patient's arm include momentary pain and/or bruising, and rarely, fainting or infection.

8.0 BENEFITS

Participants may not personally be helped by taking part in this study, but their participation may lead to knowledge that will help others. There is a potential benefit to society and future patients with prostate cancer. Participation in this study involves minimal risk and the benefits to future patients with similar conditions outweigh the risks and discomforts.

9.0 DATA HANDLING

Data will be collected in a password-protected database which will be securely stored on the Durham VA server. Only Dr. Freedland and his IRB approved study team will have access to this file. All VA consent forms and case report forms (CRFs) will be kept in a locked file cabinet housed in Dr. Freedland's locked VA research office.

De-identified data to be sent to Maragatha Kuchibhatlawill include: age, ethnicity, medical history, anthropometric measurements, laboratory results, and clinical and pathological information.

Subjects' names and identifiers will not be used when data are presented at research meetings or presented in manuscripts.

10.0 SAFETY MONITORING

The blood obtained from the blood draw will be processed at the Durham VA. Tissue samples will be labeled with the study subject number and date of surgery. Data will be labeled with the study subject number only. The key to this code will be kept on the Durham VA server with access limited to Dr. Freedland's VA research team and this code will not be released outside of the VA.

11.0 REPORTING ADVERSE EVENTS

If a severe adverse event occurs during the blood draws, this will be reported to the Duke and Durham VA IRB as required. SAEs related to anything other than the blood draws will not be documented or reported. Any unanticipated problems involving data security will also be reported to the Durham VA IRB and ISO as required.

An AE is considered "serious" if in the opinion of the investigator it is one of the following outcomes:

- Fatal
- Life-threatening
- Constitutes a congenital anomaly or birth defect
- A medically significant condition (defined as an event that compromises subject safety or may require medical or surgical intervention to prevent one of the three outcomes above).
- Requires inpatient hospitalization or prolongation of existing hospitalization
- Results in persistent or significant incapacity or substantial disruption to conduct normal life functions.

12.0 DISSEMINATION, NOTIFICATION, AND REPORTING RESULTS

The results of findings may be published in high impact factor peer-reviewed scientific journals. Therefore, all subjects may have access to the study findings. Moreover, as the results of these analyses are exploratory in nature, no effort will be made to contact each individual regarding study results.

REFERENCES

1. **Jemal A, Siegel R, Xu J, Ward E.** Cancer statistics, 2010. CA Cancer J Clin. 2010;60(5):277-300.
2. **Dillard PR, Lin MF, Khan SA.** Androgen-independent prostate cancer cells acquire the complete steroidogenic potential of synthesizing testosterone from cholesterol. Mol Cell Endocrinol. 2008;295(1-2):115-120.
3. **Mostaghel EA, Nelson PS.** Intracrine androgen metabolism in prostate cancer progression: mechanisms of castration resistance and therapeutic implications. Best Pract Res Clin Endocrinol Metab. 2008;22(2):243-258.
4. **Ang JE, Olmos D, de Bono JS.** CYP17 blockade by abiraterone: further evidence for frequent continued hormone-dependence in castration-resistant prostate cancer. Br J Cancer. 2009.
5. **Antonarakis ES, Eisenberger MA.** Is abiraterone acetate well tolerated and effective in the treatment of castration-resistant prostate cancer? Nat Clin Pract Oncol. 2009;6(1):12-13.
6. **Attard G, Reid AH, A'Hern R, Parker C, Oommen NB, Folkerd E, et al.** Selective inhibition of CYP17 with abiraterone acetate is highly active in the treatment of castration-resistant prostate cancer. J Clin Oncol. 2009;27(23):3742-3748.
7. **Attard G, Reid AH, Yap TA, Raynaud F, Dowsett M, Settatree S, et al.** Phase I clinical trial of a selective inhibitor of CYP17, abiraterone acetate, confirms that castration-resistant prostate cancer commonly remains hormone driven. J Clin Oncol. 2008;26(28):4563-4571.
8. **Leon CG, Locke JA, Adomat HH, Etinger SL, Twiddy AL, Neumann RD, et al.** Alterations in cholesterol regulation contribute to the production of intratumoral androgens during progression to castration-resistant prostate cancer in a mouse xenograft model. Prostate.70(4):390-400.
9. **Madan RA, Arlen PM.** Abiraterone. Cougar Biotechnology. IDrugs. 2006;9(1):49-55.
10. **O'Donnell A, Judson I, Dowsett M, Raynaud F, Dearnaley D, Mason M, et al.** Hormonal impact of the 17alpha-hydroxylase/C(17,20)-lyase inhibitor abiraterone acetate (CB7630) in patients with prostate cancer. Br J Cancer. 2004;90(12):2317-2325.
11. **Ryan CJ, Smith MR, Fong L, Rosenberg JE, Kantoff P, Raynaud F, et al.** Phase I clinical trial of the CYP17 inhibitor abiraterone acetate demonstrating clinical activity in patients with castration-resistant prostate cancer who received prior ketoconazole therapy. J Clin Oncol.28(9):1481-1488.
12. **Shah S, Ryan C.** Abiraterone acetate for castration resistant prostate cancer. Expert Opin Investig Drugs.
13. **Harris WP, Mostaghel EA, Nelson PS, Montgomery B.** Androgen deprivation therapy: progress in understanding mechanisms of resistance and optimizing androgen depletion. Nat Clin Pract Urol. 2009;6(2):76-85.
14. **Labrie F.** Adrenal androgens and intracrinology. Semin Reprod Med. 2004;22(4):299-309.
15. **Locke JA, Guns ES, Lubik AA, Adomat HH, Hendy SC, Wood CA, et al.** Androgen levels increase by intratumoral de novo steroidogenesis during progression of castration-resistant prostate cancer. Cancer Res. 2008;68(15):6407-6415.
16. **Mohler JL, Gregory CW, Ford OH, 3rd, Kim D, Weaver CM, Petrusz P, et al.** The androgen axis in recurrent prostate cancer. Clin Cancer Res. 2004;10(2):440-448.

17. **Reid AH, Attard G, Barrie E, de Bono JS.** CYP17 inhibition as a hormonal strategy for prostate cancer. Nat Clin Pract Urol. 2008;5(11):610-620.
18. **Singer EA, Golijanin DJ, Messing EM.** Androgen deprivation therapy for advanced prostate cancer: why does it fail and can its effects be prolonged? Can J Urol. 2008;15(6):4381-4387.
19. **Titus MA, Schell MJ, Lih FB, Tomer KB, Mohler JL.** Testosterone and dihydrotestosterone tissue levels in recurrent prostate cancer. Clin Cancer Res. 2005;11(13):4653-4657.
20. Portney LG, Watkins MP. *Foundations of clinical research: Applications to practice*. Norwalk, Conn: Appleton & Lange 1993.
21. **Talcott JA, Spain P, Clark JA, Carpenter WR, Do YK, Hamilton RJ, et al.** Hidden barriers between knowledge and behavior: the North Carolina prostate cancer screening and treatment experience. Cancer. 2007;109(8):1599-1606.
22. **Hamilton RJ, Aronson WJ, Presti JC, Jr., Terris MK, Kane CJ, Amling CL, et al.** Race, biochemical disease recurrence, and prostate-specific antigen doubling time after radical prostatectomy: results from the SEARCH database. Cancer. 2007;110(10):2202-2209.
23. **Ahn J, Kibel AS, Park JY, Rebbeck TR, Rennert H, Stanford JL, et al.** Prostate cancer predisposition loci and risk of metastatic disease and prostate cancer recurrence. Clin Cancer Res.17(5):1075-1081.
24. **Gudmundsson J, Sulem P, Manolescu A, Amundadottir LT, Gudbjartsson D, Helgason A, et al.** Genome-wide association study identifies a second prostate cancer susceptibility variant at 8q24. Nat Genet. 2007;39(5):631-637.
25. **Haiman CA, Patterson N, Freedman ML, Myers SR, Pike MC, Waliszewska A, et al.** Multiple regions within 8q24 independently affect risk for prostate cancer. Nat Genet. 2007;39(5):638-644.
26. **Wang Y, Ray AM, Johnson EK, Zuhlke KA, Cooney KA, Lange EM.** Evidence for an association between prostate cancer and chromosome 8q24 and 10q11 genetic variants in African American men: the Flint Men's Health Study. Prostate.71(3):225-231.
27. **Witte JS.** Multiple prostate cancer risk variants on 8q24. Nat Genet. 2007;39(5):579-580.
28. **Yeager M, Orr N, Hayes RB, Jacobs KB, Kraft P, Wacholder S, et al.** Genome-wide association study of prostate cancer identifies a second risk locus at 8q24. Nat Genet. 2007;39(5):645-649.
29. **Chu LW, Meyer TE, Li Q, Menashe I, Yu K, Rosenberg PS, et al.** Association between genetic variants in the 8q24 cancer risk regions and circulating levels of androgens and sex hormone-binding globulin. Cancer Epidemiol Biomarkers Prev.19(7):1848-1854.
30. **Gaston KE, Kim D, Singh S, Ford OH, 3rd, Mohler JL.** Racial differences in androgen receptor protein expression in men with clinically localized prostate cancer. J Urol. 2003;170(3):990-993.
31. **Mohler JL, Gaston KE, Moore DT, Schell MJ, Cohen BL, Weaver C, et al.** Racial differences in prostate androgen levels in men with clinically localized prostate cancer. J Urol. 2004;171(6 Pt 1):2277-2280.
32. **Singer EA, Golijanin DJ, Miyamoto H, Messing EM.** Androgen deprivation therapy for prostate cancer. Expert Opin Pharmacother. 2008;9(2):211-228.

33. **Huggins C, Hodges C.** Studies on prostatic cancer: I. The effect of castration, of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. Cancer Research. 1941;1:293-297.

34. **Fenton MA, Shuster TD, Fertig AM, Taplin ME, Kolvenbag G, Bubley GJ, et al.** Functional characterization of mutant androgen receptors from androgen-independent prostate cancer. Clin Cancer Res. 1997;3(8):1383-1388.

35. **Ross RW, Xie W, Regan MM, Pomerantz M, Nakabayashi M, Daskivich TJ, et al.** Efficacy of androgen deprivation therapy (ADT) in patients with advanced prostate cancer: association between Gleason score, prostate-specific antigen level, and prior ADT exposure with duration of ADT effect. Cancer. 2008;112(6):1247-1253.

36. **Taplin ME.** Drug insight: role of the androgen receptor in the development and progression of prostate cancer. Nat Clin Pract Oncol. 2007;4(4):236-244.

37. **Taplin ME, Balk SP.** Androgen receptor: a key molecule in the progression of prostate cancer to hormone independence. J Cell Biochem. 2004;91(3):483-490.

38. **Taplin ME, Bubley GJ, Ko YJ, Small EJ, Upton M, Rajeshkumar B, et al.** Selection for androgen receptor mutations in prostate cancers treated with androgen antagonist. Cancer Res. 1999;59(11):2511-2515.

39. **Taplin ME, Bubley GJ, Shuster TD, Frantz ME, Spooner AE, Ogata GK, et al.** Mutation of the androgen-receptor gene in metastatic androgen-independent prostate cancer. N Engl J Med. 1995;332(21):1393-1398.

40. **Taplin ME, Rajeshkumar B, Halabi S, Werner CP, Woda BA, Picus J, et al.** Androgen receptor mutations in androgen-independent prostate cancer: Cancer and Leukemia Group B Study 9663. J Clin Oncol. 2003;21(14):2673-2678.

41. **Veldscholte J, Ris-Stalpers C, Kuiper GG, Jenster G, Berrevoets C, Claassen E, et al.** A mutation in the ligand binding domain of the androgen receptor of human LNCaP cells affects steroid binding characteristics and response to anti-androgens. Biochem Biophys Res Commun. 1990;173(2):534-540.

42. **Geller J, Albert J, Loza D.** Steroid levels in cancer of the prostate--markers of tumour differentiation and adequacy of anti-androgen therapy. J Steroid Biochem. 1979;11(1B):631-636.

43. **Geller J, Albert J, Nachtsheim D, Loza D, Lippman S.** Steroid levels in cancer of the prostate--markers of tumor differentiation and adequacy of anti-androgen therapy. Prog Clin Biol Res. 1979;33:103-111.

44. **Belanger B, Belanger A, Labrie F, Dupont A, Cusan L, Monfette G.** Comparison of residual C-19 steroids in plasma and prostatic tissue of human, rat and guinea pig after castration: unique importance of extratesticular androgens in men. J Steroid Biochem. 1989;32(5):695-698.

45. **de Bono JS, Logothetis CJ, Molina A, Fizazi K, North S, Chu L, et al.** Abiraterone and increased survival in metastatic prostate cancer. N Engl J Med.364(21):1995-2005.

46. Scher HI, Logothetis C, Molina A, Goodman OB, Sternberg CN, Chi KN, et al. Improved survival outcomes in clinically relevant patient subgroups from COU-AA-301, a phase III study of abiraterone acetate (AA) plus prednisone (P) in patients with metastatic castration-resistant prostate cancer (mCRPC) progressing after docetaxel-based chemotherapy. Paper presented at: 2011 Genitourinary Cancers Symposium 2011; Orlando, FL.

47. **Danila DC, Morris MJ, de Bono JS, Ryan CJ, Denmeade SR, Smith MR, et al.** Phase II multicenter study of abiraterone acetate plus prednisone therapy in patients with docetaxel-treated castration-resistant prostate cancer. J Clin Oncol.28(9):1496-1501.
48. **Forti G, Salerno R, Moneti G, Zoppi S, Fiorelli G, Marinoni T, et al.** Three-month treatment with a long-acting gonadotropin-releasing hormone agonist of patients with benign prostatic hyperplasia: effects on tissue androgen concentration, 5 alpha-reductase activity and androgen receptor content. J Clin Endocrinol Metab. 1989;68(2):461-468.
49. **Nishiyama T, Hashimoto Y, Takahashi K.** The influence of androgen deprivation therapy on dihydrotestosterone levels in the prostatic tissue of patients with prostate cancer. Clin Cancer Res. 2004;10(21):7121-7126.
50. **Montgomery RB, Mostaghel EA, Vessella R, Hess DL, Kalhorn TF, Higano CS, et al.** Maintenance of intratumoral androgens in metastatic prostate cancer: a mechanism for castration-resistant tumor growth. Cancer Res. 2008;68(11):4447-4454.
51. **Thysell E, Surowiec I, Hornberg E, Crnalic S, Widmark A, Johansson AI, et al.** Metabolomic characterization of human prostate cancer bone metastases reveals increased levels of cholesterol. PLoS One.5(12):e14175.
52. **Haug A, Hostmark AT, Spydevold O, Eilertsen E.** Hypercholesterolaemia, hypotriacylglycerolaemia and increased lipoprotein lipase activity following orchidectomy in rats. Acta Endocrinol (Copenh). 1986;113(1):133-139.
53. **Leblanc M, Belanger MC, Julien P, Tchernof A, Labrie C, Belanger A, et al.** Plasma lipoprotein profile in the male cynomolgus monkey under normal, hypogonadal, and combined androgen blockade conditions. J Clin Endocrinol Metab. 2004;89(4):1849-1857.
54. **Pick R, Stamler J, Rodbard S, Katz LN.** Effects of testosterone and castration on cholesteremia and atherogenesis in chicks on high fat, high cholesterol diets. Circ Res. 1959;7(2):202-204.
55. **Freeman MR, Solomon KR.** Cholesterol and prostate cancer. J Cell Biochem. 2004;91(1):54-69.
56. **Schaffner CP.** Prostatic cholesterol metabolism: regulation and alteration. Prog Clin Biol Res. 1981;75A:279-324.
57. **Swyer GI.** The cholesterol content of normal and enlarged prostates. Cancer Res. 1942;2:372-375.
58. **Solomon KR, Freeman MR.** Do the cholesterol-lowering properties of statins affect cancer risk? Trends Endocrinol Metab. 2008;19(4):113-121.
59. **Platz EA, Leitzmann MF, Visvanathan K, Rimm EB, Stampfer MJ, Willett WC, et al.** Statin drugs and risk of advanced prostate cancer. J Natl Cancer Inst. 2006;98(24):1819-1825.
60. **Platz EA, Clinton SK, Giovannucci E.** Association between plasma cholesterol and prostate cancer in the PSA era. Int J Cancer. 2008;123(7):1693-1698.
61. **Blais L, Desgagne A, LeLorier J.** 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors and the risk of cancer: a nested case-control study. Arch Intern Med. 2000;160(15):2363-2368.
62. **Bravi F, Scotti L, Bosetti C, Talamini R, Negri E, Montella M, et al.** Self-reported history of hypercholesterolaemia and gallstones and the risk of prostate cancer. Ann Oncol. 2006.

63. **Freeman MR, Solomon KR, Moyad M.** Statins and the risk of cancer. Jama. 2006;295(23):2720-2721; author reply 2721-2722.

64. **Murtola TJ, Tammela TL, Lahtela J, Auvinen A.** Cholesterol-Lowering Drugs and Prostate Cancer Risk: A Population-based Case-Control Study. Cancer Epidemiol Biomarkers Prev. 2007;16(11):2226-2232.

65. **Pedersen TR, Wilhelmsen L, Faergeman O, Strandberg TE, Thorgeirsson G, Troedsson L, et al.** Follow-up study of patients randomized in the Scandinavian simvastatin survival study (4S) of cholesterol lowering. Am J Cardiol. 2000;86(3):257-262.

66. **Platz E, Leitzmann M, Visvanathan K, al. e.** Cholesterol-lowering drugs including statins and the risk of prostate cancer in a large prospective cohort study. *Proc Amer Assoc Cancer Res*2005.

67. Platz EA, Clinton SK, Giovannucci E. Association between plasma cholesterol and prostate cancer. Paper presented at: AACR Frontiers in Cancer Prevention Research 2006; Boston, MA.

68. **Platz EA, Leitzmann MF, Visvanathan K, Stampfer MJ, Willett WC, Rimm EB, et al.** Cholesterol-lowering drugs including statins and the risk of prostate cancer in a large prospective cohort study. AACR Meeting Abstracts. 2005;2005(1):1034-a-.

69. **Zhuang L, Kim J, Adam RM, Solomon KR, Freeman MR.** Cholesterol targeting alters lipid raft composition and cell survival in prostate cancer cells and xenografts. J Clin Invest. 2005;115(4):959-968.

70. **Zhuang L, Lin J, Lu ML, Solomon KR, Freeman MR.** Cholesterol-rich lipid rafts mediate akt-regulated survival in prostate cancer cells. Cancer Res. 2002;62(8):2227-2231.

71. **Iso H, Ikeda A, Inoue M, Sato S, Tsugane S.** Serum cholesterol levels in relation to the incidence of cancer: the JPHC study cohorts. Int J Cancer. 2009;125(11):2679-2686.

72. **Michaud DS, Augustsson K, Rimm EB, Stampfer MJ, Willet WC, Giovannucci E.** A prospective study on intake of animal products and risk of prostate cancer. Cancer Causes Control. 2001;12(6):557-567.

73. **Fleshner N, Bagnell PS, Klotz L, Venkateswaran V.** Dietary fat and prostate cancer. J Urol. 2004;171(2 Pt 2):S19-24.

74. **Kolonel LN, Nomura AM, Cooney RV.** Dietary fat and prostate cancer: current status. J Natl Cancer Inst. 1999;91(5):414-428.

75. **McCarty MF.** Mortality from Western cancers rose dramatically among African-Americans during the 20th century: are dietary animal products to blame? Med Hypotheses. 2001;57(2):169-174.

76. **McCredie M, Williams S, Coates M.** Cancer mortality in East and Southeast Asian migrants to New South Wales, Australia, 1975-1995. Br J Cancer. 1999;79(7-8):1277-1282.

77. **Pu YS, Chiang HS, Lin CC, Huang CY, Huang KH, Chen J.** Changing trends of prostate cancer in Asia. Aging Male. 2004;7(2):120-132.

78. **Walker M, Aronson KJ, King W, Wilson JW, Fan W, Heaton JP, et al.** Dietary patterns and risk of prostate cancer in Ontario, Canada. Int J Cancer. 2005;116(4):592-598.

79. **Wu K, Hu FB, Willett WC, Giovannucci E.** Dietary patterns and risk of prostate cancer in U.S. men. Cancer Epidemiol Biomarkers Prev. 2006;15(1):167-171.

80. **Baigent C, Keech A, Kearney PM, Blackwell L, Buck G, Pollicino C, et al.** Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90,056 participants in 14 randomised trials of statins. Lancet. 2005;366(9493):1267-1278.

81. **Browning DR, Martin RM.** Statins and risk of cancer: a systematic review and metaanalysis. Int J Cancer. 2007;120(4):833-843.

82. **Dale KM, Coleman CI, Henyan NN, Kluger J, White CM.** Statins and cancer risk: a meta-analysis. Jama. 2006;295(1):74-80.

83. **Duncan RE, El-Sohemy A, Archer MC.** Statins and the risk of cancer. Jama. 2006;295(23):2720; author reply 2721-2722.

84. **Flick ED, Habel LA, Chan KA, Van Den Eeden SK, Quinn VP, Haque R, et al.** Statin Use and Risk of Prostate Cancer in the California Men's Health Study Cohort. Cancer Epidemiol Biomarkers Prev. 2007;16(11):2218-2225.

85. **Graaf MR, Beiderbeck AB, Egberts AC, Richel DJ, Guchelaar HJ.** The risk of cancer in users of statins. J Clin Oncol. 2004;22(12):2388-2394.

86. **Jacobs EJ, Rodriguez C, Bain EB, Wang Y, Thun MJ, Calle EE.** Cholesterol-lowering drugs and advanced prostate cancer incidence in a large u.s. Cohort. Cancer Epidemiol Biomarkers Prev. 2007;16(11):2213-2217.

87. **Salinas CA, Agalliu I, Stanford JL, Lin DW.** Statins and the risk of cancer. Jama. 2006;295(23):2721; author reply 2721-2722.

88. **Mondul AM, Clipp SL, Helzlsouer KJ, Platz EA.** Association between plasma total cholesterol concentration and incident prostate cancer in the CLUE II cohort. Cancer Causes Control.21(1):61-68.

89. **Mondul AM, Selvin E, De Marzo AM, Freedland SJ, Platz EA.** Statin drugs, serum cholesterol, and prostate-specific antigen in the National Health and Nutrition Examination Survey 2001-2004. Cancer Causes Control.

90. **Solomon KR, Pelton K, Boucher K, Joo J, Tully C, Zurakowski D, et al.** Ezetimibe is an inhibitor of tumor angiogenesis. Am J Pathol. 2009;174(3):1017-1026.

91. **Gao H, Ouyang X, Banach-Petrosky WA, Shen MM, Abate-Shen C.** Emergence of androgen independence at early stages of prostate cancer progression in Nkx3.1; Pten mice. Cancer Res. 2006;66(16):7929-7933.

92. **Adam RM, Mukhopadhyay NK, Kim J, Di Vizio D, Cinar B, Boucher K, et al.** Cholesterol sensitivity of endogenous and myristoylated Akt. Cancer Res. 2007;67(13):6238-6246.

93. **Tang W, Ma Y, Yu L.** Plasma cholesterol is hyperresponsive to statin in ABCG5/ABCG8 transgenic mice. Hepatology. 2006;44(5):1259-1266.

94. **Endo A.** Chemistry, biochemistry, and pharmacology of HMG-CoA reductase inhibitors. Klin Wochenschr. 1988;66(10):421-427.

95. **Lichtman AH, Clinton SK, Iiyama K, Connelly PW, Libby P, Cybulsky MI.** Hyperlipidemia and atherosclerotic lesion development in LDL receptor-deficient mice fed defined semipurified diets with and without cholate. Arterioscler Thromb Vasc Biol. 1999;19(8):1938-1944.

96. **Paigen B.** Genetics of responsiveness to high-fat and high-cholesterol diets in the mouse. Am J Clin Nutr. 1995;62(2):458S-462S.

97. **Parhami F, Tintut Y, Beamer WG, Gharavi N, Goodman W, Demer LL.** Atherogenic high-fat diet reduces bone mineralization in mice. J Bone Miner Res. 2001;16(1):182-188.
98. **Freedland SJ, Mavropoulos J, Wang A, Darshan M, Demark-Wahnefried W, Aronson WJ, et al.** Carbohydrate restriction, prostate cancer growth, and the insulin-like growth factor axis. Prostate. 2008;68(1):11-19.
99. **Mavropoulos JC, Buschemeyer WC, 3rd, Tewari AK, Rokhfeld D, Pollak M, Zhao Y, et al.** The effects of varying dietary carbohydrate and fat content on survival in a murine LNCaP prostate cancer xenograft model. Cancer Prev Res (Phila Pa). 2009;2(6):557-565.
100. **Altmann SW, Davis HR, Jr., Zhu LJ, Yao X, Hoos LM, Tetzloff G, et al.** Niemann-Pick C1 Like 1 protein is critical for intestinal cholesterol absorption. Science. 2004;303(5661):1201-1204.
101. **Davis HR, Jr., Zhu LJ, Hoos LM, Tetzloff G, Maguire M, Liu J, et al.** Niemann-Pick C1 Like 1 (NPC1L1) is the intestinal phytosterol and cholesterol transporter and a key modulator of whole-body cholesterol homeostasis. J Biol Chem. 2004;279(32):33586-33592.
102. **Jurado J, Seip R, Thompson PD.** Effectiveness of ezetimibe in clinical practice. Am J Cardiol. 2004;93(5):641-643.
103. **Knopp RH, Gitter H, Truitt T, Bays H, Manion CV, Lipka LJ, et al.** Effects of ezetimibe, a new cholesterol absorption inhibitor, on plasma lipids in patients with primary hypercholesterolemia. Eur Heart J. 2003;24(8):729-741.